

REMARKS

Claims 1, 5 and 16 are amended to recite that the oxidizing agent is a chemical oxidizing agent selected from salts. Support for the amendments can be found, for example, at page 11 of the present specification. New claims 42-46 depend from claims 1, 5 and 16, respectively, and further define the oxidizing agent. Support for claims 42-46 can be found, for example, at page 11 of the present specification. No new matter is added. Upon entry of the Amendment, which is respectfully requested, claims 1-46 will be pending.

Response to Claim Rejections under 35 U.S.C. § 112

At page 7 of the Office Action, claims 22 and 40 are rejected under 35 U.S.C. § 112, second paragraph, as being indefinite. According to the Examiner, the claims lack a definition for enzyme activity.

Applicants traverse and respectfully request the Examiner to reconsider in view of the following remarks.

In the present invention, the laccase activity was determined using ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)) as a substrate. The activity of laccase was measured at room temperature using pH 4.5 as the determination pH and expressed in nanokatals (nkat). The SI unit of catalytic activity is defined as the amount of enzyme activity that converts 1 mol of substrate per second in the assay conditions. The specific conditions of each chemical reaction are described in the working examples so that enzyme activity can be calculated in katal for each condition.

In view of the foregoing, a person having ordinary skill in the art would be able to ascertain the definition of enzyme activity with respect to the present invention. The document

by Niku-Paavola et al. [1988]¹ is enclosed herewith to provide evidence of what is known in the art. In the document by Niku-Paavola et al. [1988], laccase activity is calculated in the same way as that of the present invention.

In view of the above, it is respectfully requested that the § 112, second paragraph, of claims 22 and 40 be withdrawn.

Response to Claim Rejections under 35 U.S.C. § 102

Claims 1-6, 9-15, 22-29, 31-34 and 41

At page 8 of the Office Action, claims 1-6, 9-15, 22-29, 31-34 and 41 are rejected under 35 U.S.C. § 102(e) as allegedly being anticipated by U.S. Patent Application Publication No. 2003/0186036 (Goodell).

Applicants traverse and respectfully request the Examiner to reconsider in view of the following remarks.

In order to anticipate a claim under 35 U.S.C. § 102, a reference must disclose within the four corners of the document not only all of the elements claimed but also all of the elements arranged or combined in the same way as recited in the claim. *Net MoneyIn, Inc. v. Verisign, Inc.*, 2008 U.S. App. LEXIS 21827, 1, 27 (Fed. Cir. 2008).

In this case, Goodell fails to disclose a specific example which meets the modifying agent as required in the presently claimed invention. A person having ordinary skill in the art would have to pick and choose amongst the various exemplary chelators of Goodell to arrive at one of

¹ Niku-Paavola M-L, Karhunen E, Salola P, Raunio V. Lignolytic enzymes of the white-rot fungus *Phlebia radiata*. *Biochem. J.* 1988:254; 877-884 is enclosed herewith.

querctin and kaemferol. Goodell does not teach all elements of the present claims and can not be said to anticipate the present invention.

Further, Goodell described the oxidation of lignocellulosic materials and organic compounds using hydroxyl radicals that are generated in mediated Fenton reactions. The aim of Goodell is to produce an efficient oxidation achieved with said hydroxyl radicals. The purpose of the mediator of Goodell is to bind metals and then “be reduced back to its reduced form” (page 6, [0093]). These “cycling” mediators are selected from compounds that do not exit the system by binding to a starting material or the product. Instead, the “cycling” mediators are selected from compounds that can be regenerated and reused. Therefore, the redox cycling chelators of Goodell do not expressly or inherently act as modifying agents as required by the presently claimed invention.

Further, even if the mediators of Goodell were to bind to the fibre, they would not provide the fibre with the desired functionalization.

Goodell does not expressly or inherently disclose each and every element of the presently claimed invention, and thus, does not anticipate the presently claimed invention. Accordingly, withdrawal of the § 102 rejection of claims 1-6, 9-15, 22-29, 31-34 and 41 based on Goodell is respectfully requested.

Claims 1-6, 9-14, 23-33 and 41

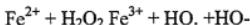
At page 10 of the Office Action, claims 1-6, 9-14, 23-33 and 41 are rejected under 35 U.S.C. § 102(b) as allegedly being anticipated by U.S. Patent No. 6,136,041 (Jaschinski).

Applicants traverse and respectfully request the Examiner to reconsider in view of the following remarks.

The Examiner states that Jaschinski discloses hydrogen peroxide [claim 3]; hydrogen peroxide is the same oxidant as used in the present invention.

The claims are amended to recite that the oxidizing agent of the present invention is a chemical oxidizing agent selected from salts. Further, in the present invention, the targeted reaction is the coupling of a phenoxy radical of a phenolic species to the fibre surface. The oxidizing agent is a chemical oxidizing agent selected from salts and is used as an activator.

Jaschinski relates to chelator compounds, which can bind to a transition metal catalyst to decompose hydrogen peroxide to hydroxyl radicals according to the scheme:



The activator of the present invention is different from the species used or formed in the above reaction. For example, the APS mentioned as an exemplary oxidizing agent in the present application reacts through a sulphate radical.

In view of the above, it is submitted that the Jaschinski does not anticipate the present invention. Accordingly, withdrawal of the § 102 rejection of claims 1-6, 9-14, 23-33 and 41 based on Jaschinski is respectfully requested.

Claims 1-14, 16-33, 35-39 and 41

At page 12 of the Office Action, claims 1-14, 16-33, 35-39 and 41 are rejected under 35 U.S.C. § 102(b) as allegedly being anticipated by U.S. Patent No. 6,187,136 (Pederson).

Applicants traverse and respectfully request the Examiner to reconsider in view of the following remarks.

The present invention provides a way to functionalize the lignocellulosic fibres which, in turn, provides the fibres with properties foreign to the native fibre. More specifically, in the present invention, the oxidized fibre material is contacted with a modifying agent containing at

least one first functional portion, which is compatible with the oxidized fibre material. The modifying agent is capable of providing the lignocellulosic fibre material with properties foreign to the native fibre.

Pederson does not disclose that the modified fiber is polymerized in the presence of an oxidizing agent in such a way that one end of the polymer chain is attached to the primed matrix of the fiber, as recited in the present invention.

Further, Pedersen does not describe the process or the aims of the present invention. Pederson describes a process for the enzyme-assisted grafting of small negatively charged phenolic compounds, such as carboxylic acids, to lignocellulosic material (see, for example, col. 5, lines 21-24). Pederson discloses that the modified lignocellulosic material is treated with a cationic strengthening agent having an ionic charge of sign opposite to that which is conferred on the modified lignocellulosic material (see col. 8, lines 62-65 to col. 9, lines 1-3). The aim of Pederson is to achieve an improvement of the bond strength.

In view of the above, it is submitted that Pederson fails to anticipate the presently claimed invention. Accordingly, withdrawal of the § 102 rejection of claims 1-14, 16-33, 35-39 and 41 based on Pederson is respectfully requested.

Response to Claim Rejections under 35 U.S.C. § 103

Claims 22 and 40

At page 15 of the Office Action, claims 22 and 40 are rejected under 35 U.S.C. § 102(b) as allegedly being anticipated by or, in the alternative, under 35 U.S.C. § 103(a) as allegedly being obvious over Pederson.

Applicants traverse and respectfully request the Examiner to reconsider in view of the following remarks.

The Examiner's position on this rejection raises the same issues as presented by the § 112, second paragraph, rejection set forth above, and thus, it is respectfully requested that the rejection be withdrawn for the reasons set forth in response to the § 112 rejection.

In particular, in the present invention, the laccase activity was determined as described above, and under the assay conditions at which the enzyme activity was measured, the ranges of Pederson do not overlap with those of the present invention.

Accordingly, withdrawal of the § 102(b) or, in the alternative, § 103(a) rejection of claims 22 and 40 based on Pederson is respectfully requested.

New Claims 42-46

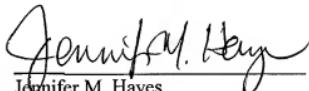
New claims 42-46 depend from claims 1, 5 and 16, respectively, and are patentable at least by virtue of their dependencies from claims 1, 5 and 16 which are patentable for the reasons set forth above.

Conclusion

In view of the above, reconsideration and allowance of this application are now believed to be in order, and such actions are hereby solicited. If any points remain in issue which the Examiner feels may be best resolved through a personal or telephone interview, the Examiner is kindly requested to contact the undersigned at the telephone number listed below.

The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

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Ligninolytic enzymes of the white-rot fungus *Phlebia radiata*

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One oxidase (EC 1.10.3.2) and three lignin peroxidases (EC 1.11.1.-) were purified from the culture liquid of the white-rot fungus *Phlebia radiata* Fr. All the enzymes were glycoproteins. The oxidase had M_r 64000 and the lignin peroxidases I, II and III had M_r values 42000, 45000 and 44000 respectively. The lignin peroxidases were found to share common antigenic determinants: lignin peroxidases II and III were serologically indistinguishable and lignin peroxidase I was related but distinguishable. The oxidase did not share any immunological properties with the lignin peroxidases. Lignin peroxidases of *Phlebia* contain protoporphyrin IX as a prosthetic group. In the presence of H_2O_2 and an electron donor, veratryl alcohol, lignin peroxidases exhibit spectral shifts analogous to those of animal catalase (EC 1.11.1.6). *Phlebia* enzymes show optimal activity at pH 3-4.5 at 40 °C and are stable in the pH range 5-6. They modify Kraft lignin and phenolic compounds containing hydroxy and methoxy groups.

INTRODUCTION

Oxidative extracellular enzymes have been shown to be in part responsible for the degradation of lignin by the white-rot fungus *Phanerochaete chrysosporium* (Tien & Kirk, 1983). These enzymes have similarities to certain peroxidases (EC 1.11.1.5-EC 1.11.1.8) insofar as the prosthetic group and reaction mechanisms are concerned (Tien *et al.*, 1986; Andersson *et al.*, 1987). Characterization of the gene coding for the major ligninolytic component of *Phanerochaete* revealed homology in the amino acid sequence of lignin peroxidase with the other peroxidases (Tien & Tu, 1987). Homology is found near the residues essential for peroxidase activity.

Among the characteristics of ligninolytic enzymes so far studied are the cleavage of aromatic rings in lignin model compounds (Leisola *et al.*, 1985) and degradative oxidation of non-phenolic compounds such as the environmental pollutant benzo[a]pyrene (Haenmerli *et al.*, 1986). The possible application of these enzymes in a range of different fields has stimulated much research into the biochemistry of the white-rot fungi.

Hitherto, most studies have concerned the enzymes of *Phanerochaete*, although the oxidative extracellular enzyme system is probably not identical in all white-rot fungi. Differences can be seen, for example, in the efficiency of lignin degradation (Hatakka & Uusi-Rauva, 1983) and in the pattern of degradation products (Ander *et al.*, 1980). In order to learn more about the biochemical function of ligninolytic enzymes in general, the purified enzymes of a number of different white-rot fungi should be studied.

The white-rot fungus *Phlebia radiata* has been shown to degrade lignin even more effectively than *Phanerochaete chrysosporium* under certain experimental conditions (Hatakka & Uusi-Rauva, 1983). The extracellular ligninolytic enzymes of *Phlebia* were shown to include three enzymes oxidizing veratryl alcohol and one oxidizing an oxidation-reduction indicator, ABTS stain (Niku-Paavola, 1987).

We present here the purification and properties of these four enzymes.

MATERIALS AND METHODS

Enzyme production

The organism used was *Phlebia radiata* Fr. strain 79 (kindly supplied by A. Hatakka, Department of Microbiology, University of Helsinki). Cultivations were performed in 1-litre batches in a laboratory bioreactor (Hatakka *et al.*, 1987). The low-nitrogen medium described by Fenn & Kirk (1979) was used with slight modifications. Vitamins and mineral salts were omitted, glucose and guaiacol (Merck, Darmstadt, Germany) concentrations were 2.8 mM and 0.8 M respectively and 0.05% (w/v) Tween 80 (Atlas, Essen, Germany) was included. Oxidase was produced with the use of this medium with a cultivation time of 3 days. For the production of lignin peroxidases the nitrogen supply was 0.38 g of yeast extract (Difco Laboratories, Detroit, MI, U.S.A.)/l and the cultivation time was 6 days.

Enzyme purification

Culture filtrates were frozen to -20 °C for at least 20 h. Thawed liquids were filtered through glass-fibre filters (GF/C; Whatman, Maidstone, Kent, U.K.) and equilibrated with 50 mM-sodium acetate buffer, pH 5.5. Proteins were adsorbed on 15 ml of Sepharose Q Fast Flow anion-exchanger (Pharmacia, Uppsala, Sweden) in 50 mM-sodium acetate buffer, pH 5.5. Unadsorbed proteins were washed with 100 ml of buffer. Lignin peroxidase I (L_1) was eluted with 120 ml of 0.13 M-NaCl in 50 mM-sodium acetate buffer, pH 5.5. A linear gradient of 0.18-0.3 M-NaCl in 180 ml of 50 mM-sodium acetate buffer, pH 5.5, was used to elute oxidase and lignin peroxidases II and III (L_{II} and L_{III}). Active fractions were dialysed against 50 mM-sodium acetate buffer, pH 5.5, and used for characterization of enzyme components.

Abbreviations used: ABTS, 2,2-azinodi-3-ethylbenzothiazoline-6-sulphuric acid; O_2 , oxidase, L_1 , L_{II} and L_{III} , lignin peroxidases I, II and III respectively.

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Lignin peroxidase activity was determined as the ability of the enzyme to convert veratryl alcohol (Fluka, Buchs, Switzerland) into veratraldehyde in the presence of H_2O_2 (Merck) (Tien & Kirk, 1984). The buffer used was 50 mM-glycine/HCl, pH 3.0, and the temperature was 20 °C. The reaction mixture contained 3.5 μ mol of veratryl alcohol, 0.44 μ mol of H_2O_2 and 1–10 μ g of enzyme in 3 ml. The reaction was monitored during 10 min at 310 nm and activity was calculated as katalas. ϵ for veratraldehyde is 9256 $M^{-1} \cdot cm^{-1}$. Oxidase activity was assayed with ABTS (Boehringer, Mannheim, Germany) as substrate (Werner *et al.*, 1970) in 50 mM-glycine/HCl buffer, pH 3.0, at a temperature of 20 °C. The reaction mixture contained 14 μ mol of ABTS and 0.1–1 μ g of enzyme in 3 ml. The reaction was monitored during 5 min at 436 nm and activity was expressed as katalas. ϵ for the product is 29300 $M^{-1} \cdot cm^{-1}$.

Protein concentration was determined by precipitating the preparations with 5% (w/v) trichloroacetic acid, dissolving the precipitate in 2% (w/v) Na_2CO_3 solution containing 0.4% NaOH and measuring A_{280} . Human serum albumin (Sigma Chemical Co., St. Louis, MO, U.S.A.) was treated in a similar way as a reference.

The homogeneity and molecular sizes of the purified enzymes were assessed by performing electrophoresis at pH 8.5 in 25 mM-Tris/192 mM-glycine/0.1% (w/v) SDS buffer at room temperature on 10% (w/v) polyacrylamide gel containing 0.1% (w/v) SDS (Laemmli, 1970). Pharmacia low-*M*_w calibration mixture was used as reference. Proteins were localized by silver staining (Bio-Rad Laboratories, Richmond, CA, U.S.A.) (Merril *et al.*, 1981).

Antisera

Antisera were prepared against all the extracellular proteins from concentrated culture liquid and against the four purified enzymes.

During an immunization of 6 months, 1.0 mg of proteins emulsified with 1 ml of complete Freund's adjuvant (Difco Laboratories) was injected intracutaneously once a month into rabbits. The sera were collected and used without further purification for immunological tests.

Immunoelectrophoresis

Immunoelectrophoresis was performed on agarose gel in 50 mM-barbital buffer, pH 8.2, at room temperature during 45 min with a voltage gradient of 6 V/cm (Grabar & Burtin, 1964).

Glycoprotein test

Purified enzymes were treated with endoglycosidase H [endo- β -N-acetylglucosaminidase (EC 3.2.1.96) from *Streptomyces griseus* (Seikagaku Kogyo Co., Tokyo, Japan)] and endoglycosidase F [endo- β -N-acetylglucosaminidase from *Flavobacterium meningosepticum* (NEN, Boston, MA, U.S.A.)] to eliminate the possible carbohydrate moiety. Purified enzymes (1–2 μ g) were treated with 0.5 munit (12.5 ng) of endoglycosidase H in 125 mM-sodium citrate buffer, pH 5.0, at 37 °C for 24 h (Trimble & Maley, 1984). Ligninolytic enzymes were also treated with 125 munits of endoglycosidase F in 0.1 mM-sodium phosphate buffer, pH 6.1, containing 50 mM-EDTA and 0.5% (v/v) Nonidet P-40 (Sigma Chemical Co.) at 37 °C for 24 h (Elder & Alexander, 1982).

After endoglycosidase treatment enzymes were electro-

phoresed on 10% (w/v) polyacrylamide gel in denaturing conditions (Laemmli, 1970) and electroforetically transferred at pH 8.3 in 25 mM-Tris/192 mM-glycine/10% (v/v) methanol buffer for 3 h at 0.6 A (Towbin *et al.*, 1979) on nitrocellulose filters (BA 85; Schleicher und Schüll, Dassel, Germany). The filters were treated with antisera (dilution 1:1000) against *Phlebia* proteins. Detection of the protein-antibody complex was carried out by using a second antibody linked with alkaline phosphatase (EC 3.1.3.1) (Proto Blot Promega Biotech, Madison, WI, U.S.A.). Phosphatase activity was assayed with 0.1 mM-Tris/HCl buffer, pH 9.5, containing 0.1 mM-NaCl and 5 mM-MgCl₂ and with 5-bromo-4-chloroindol-3-yl phosphate as substrate and Nitro Blue Tetrazolium (Promega) for colour development.

Enzyme characteristics

The effects of hydroxylamine, NaN_3 , KCN, EDTA and NaF (all reagents from Merck) and of dithiothreitol (Sigma Chemical Co.) on enzyme activity were studied with the use of supplements in 0.1–0.001 mM concentrations. The effect of CO was studied by saturating the enzyme solutions with CO gas, corresponding to a concentration of 1 mM.

The absorption spectra of purified enzyme components were measured in 50 mM-acetate buffer, pH 5.5. The specific shifts were analysed by using supplements of 0.15 mM- H_2O_2 and 1.2 mM-veratryl alcohol added successively, as in the lignin peroxidase assay. The effects of 0.1 mM-dithiothreitol, -KCN and - NaN_3 were analysed separately.

The action of purified enzymes on monophenols and Kraft pine lignin (Indulin; AT West Waco, Covington, VA, U.S.A.) were analysed in 50 mM-glycine/HCl buffer, pH 3.0, in the presence of 0.15 mM- H_2O_2 with lignin peroxidases, and in 50 mM-2,2-dimethylsuccinate (Fluka) buffer, pH 4.5, with H_2O_2 omitted with oxidase. The action of oxidase was also analysed on L-tyrosine (Merck) and D,L-3,4-dihydroxyphenylalanine (Aldrich Chemical Co., Milwaukee, WI, U.S.A.). The incubation mixture contained 10 μ g of enzyme and 1 mg of substrate in 3 ml of buffer. The effects of enzyme were analysed after incubation at 20 °C for 10 min. The reaction mixtures were centrifuged and the supernatants were scanned from 190 to 600 nm to determine the differences between the untreated substrates and substrates treated with enzyme. H.p.l.c. analysis was performed with a Waters 6000A apparatus (Milford, MA, U.S.A.) equipped with a Lambda Max 480 u.v. detector. The column used was a 3.9 mm internal diam. \times 15 cm one of Nova-Pak C₁₈ (5 μ m particle size; Waters). The eluent contained acetonitrile/water/tetramethylammonium hydroxide/phosphoric acid (20:80:1:1, by vol.). The run was performed at 40 °C and detection was by measuring A_{240} . The following monophenols were used as references and as substrates: catechol (Koch-Light, Colnbrook, Bucks, U.K.), coumaric acid (Fluka), coniferyl alcohol (Fluka), ferulic acid (Fluka), guaiacol (Merck), vanillin (BDH Chemicals, Poole, Dorset, U.K.), veratraldehyde (Fluka), veratryl alcohol (Fluka) and veratric acid (Fluka).

RESULTS

Under the nitrogen-limited cultivation conditions *Phlebia radiata* secreted three enzymes oxidizing veratryl alcohol and one oxidizing ABTS. On the basis of their

Table 1. Purification of *Phlebia* oxidase

Purification was performed from 1 litre of 3-day-old-culture liquid in which the activity of oxidase was 4.9 nkat/ml and that of lignin peroxidase was 0.007 nkat/ml. For experimental details see the text.

Purification stage	Volume (ml)	Protein (mg/ml)	Total protein		Activity		Total activity	
			(mg)	(% recovery)	(nkat/ml)	(nkat/mg)	(nkat)	(% recovery)
Culture filtrate	980	0.025	24.5	100	4.9	196	4802	100
Purified oxidase	40	0.23	9.2	38	99.0	430	3960	82

Table 2. Purification of *Phlebia* lignin peroxidases

Purification was performed from 1 litre of 6-day-old-culture liquid in which oxidase activity was 0.428 nkat/ml and that of lignin peroxidase was 0.744 nkat/ml. For experimental details see the text.

Purification stage	Volume (ml)	Protein (mg/ml)	Total protein		Activity		Total activity	
			(mg)	(% recovery)	(nkat/ml)	(nkat/mg)	(nkat)	(% recovery)
Culture filtrate	900	0.03	27	100	0.74	25.0	669	100
L _I	10	0.68	6.8	25	1.4	2	14	2
L _{II}	37	0.18	6.6	24	4.3	24	159	24
L _{III}	48	0.17	8.1	30	5.0	30	239	36
			Σ79				Σ62	

chromatographic separation they were called lignin peroxidase I (L_I), oxidase (O_x), lignin peroxidase II (L_{II}) and lignin peroxidase III (L_{III}) (Niku-Paavola, 1987). To aid the purification, oxidase was separated from 3-day-old-culture filtrates, in which the lignin peroxidase activity was still low. The production of lignin peroxidases was enhanced when yeast extract was used as nitrogen source. After 6 days of cultivation the oxidase activity was low, whereas lignin peroxidase activities were high. The purification procedures are summarized in Tables 1 and 2. The purification increased the specific activity of oxidase 2-fold and the recovery of activity was 82%. The specific activities of L_{II} and L_{III} were not improved during the purification and that of L_I was even decreased. The total recovery of activity was 62% and that of total protein was 79%. The corresponding values for L_I were 2% and 25% respectively. Inactivation of lignin peroxidase during purification has also been reported for *Phanerochaete* enzymes (Tien *et al.*, 1986). The dissociation of haem in the purification conditions at pH 5.5 could be one reason for this. Fig. 1(a) shows that there are comparatively few proteins in the culture liquid; thus major increases in specific activities were not expected during purification.

Separated enzymes were shown to be homogeneous in denaturing SDS/polyacrylamide-gel electrophoresis. M_r values were 42000 for L_I, 64000 for O_x, 45000 for L_{II} and 44000 for L_{III}.

Endoglucosidase H treatment decreased the molecular sizes of all four *Phlebia* enzymes (Figs. 1b and 1c). Endoglucosidase F had a similar effect. The decrease in the M_r values was 5–14%. Western blotting was used to monitor the M_r values. This method is more sensitive than conventional protein staining methods and shows even minor changes quite accurately. Furthermore,

antisera will react only with the antigens in question and not with the endoglucosidases used in hydrolysis.

Immuno blotting and immunoelectrophoretic analysis showed that O_x does not react with L_I antibodies and vice versa. O_x shows non-identity with L_{II} and L_{III} (Fig. 2a). L_I, L_{II} and L_{III} shared common antigenic determinants reacting with antibodies raised against the others. L_I shows partial identity with L_{II} and L_{III} (Figs. 2b and 2c). L_{II} and L_{III} are serologically indistinguishable (Fig. 2d).

Lignin peroxidases and oxidases were inhibited by conventional inhibitors of the respiratory chain, cyanide and azide, and also by the reducing agents dithiothreitol and hydroxylamine. Fluoride was shown to be an inhibitor of oxidase but not of lignin peroxidases. EDTA and CO affected lignin peroxidases but not oxidase.

The haemoprotein nature of *Phlebia* lignin peroxidases was seen in the absorption spectra of native enzymes and their derivatives (Fig. 3), which strongly resembled those of catalase (EC 1.11.1.6) (Nicholls & Schonbaum, 1963). Native L_{III} has a Soret peak at 408 nm and minor bands at 510 and 630 nm. The maxima for all the lignin peroxidases were found within ± 5 nm of the corresponding peaks of L_{III}. Sequential addition of H₂O₂ and veratryl alcohol caused spectral shifts (Fig. 4) analogous to those of catalase peroxide Compounds I and III (Nicholls & Schonbaum, 1963). Oxidase did not show any absorption maxima at wavelengths longer than 300 nm.

The effect of the ligninolytic enzymes of *Phlebia radiata* on Kraft lignin is shown in Fig. 5. O_x and L_I most clearly modified lignin. This was seen in the absorption spectrum of the buffer-soluble fraction of lignin after the action of enzymes. In h.p.l.c. analysis, no specific monomeric component could be shown to be responsible for this

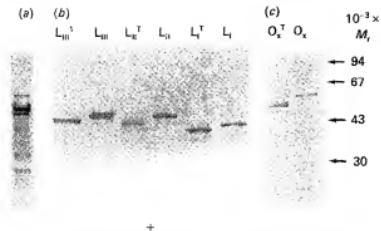


Fig. 1. Elimination of carbohydrates from *Phlebia* enzymes

(a) Culture liquid of *Phlebia*: 10 µg of protein from a 6-day-old culture. Separation was by SDS/polyacrylamide-gel electrophoresis at pH 8.5 with Tris/HCl buffer. Proteins were transferred on to a nitrocellulose filter at pH 8.3 with Tris/HCl buffer, and this was followed by incubation at pH 8.0 with Tris/HCl buffer with antiserum against all *Phlebia* proteins from 6-day-old culture liquid. The antiserum was diluted 1:1000. Thereafter incubation was with anti-(rabbit IgG) antibody conjugated with alkaline phosphatase. Immunocomplexes were detected by determining the activity of alkaline phosphatase at pH 9.5 with Tris/HCl buffer with 5-bromo-4-chloroindol-3-yl phosphate as substrate and Nitro Blue Tetrazolium for colour development. (b) Purified enzymes L₁, L₂ and L₃, 1 µg in each case, compared with 1 µg of each treated with 12.5 ng of endoglycosidase H, L₁^T, L₂^T and L₃^T respectively. SDS/polyacrylamide-gel electrophoresis, immunoblotting and detection with anti-L₁ serum were as indicated for (a). (c) Oxidase, O_x, and O_x^T treated with endoglycosidase H, O_x^T. SDS/polyacrylamide-gel electrophoresis, immunoblotting and detection with anti-O_x serum were as indicated for (a). Pharmacia low-*M*_r standards were separated and stained on the polyacrylamide gel before blotting, and their positions are indicated at the right-hand side of the Figure.

modification. However, preliminary analysis of the insoluble Kraft lignin residues from the corresponding reaction mixtures indicated slight decreases in the molecular sizes of lignin treated with enzymes. T.l.c. analysis further confirmed the release of unidentified components from lignin by the action of enzymes. Monophenols containing extra methoxy or hydroxy groups were most affected by oxidase and lignin peroxidases. In h.p.l.c. analysis, this was seen both as the disappearance of the original substrate and as the appearance of new, unidentified, components (Fig. 6). *Phlebia* oxidase did not affect tyrosine or 3,4-dihydroxyphenylalanine.

DISCUSSION

Three lignin peroxidases and one oxidase were purified from the culture filtrate of the white-rot fungus *Phlebia radiata*. Under similar nitrogen-limited cultivation conditions *Phanerochaete* has been shown to secrete at least six lignin peroxidases (Kirk *et al.*, 1986) and various Mn-peroxidases affecting lignin model compounds (Kuwahara *et al.*, 1984). Oxidase has not been purified from *Phanerochaete* cultivations.

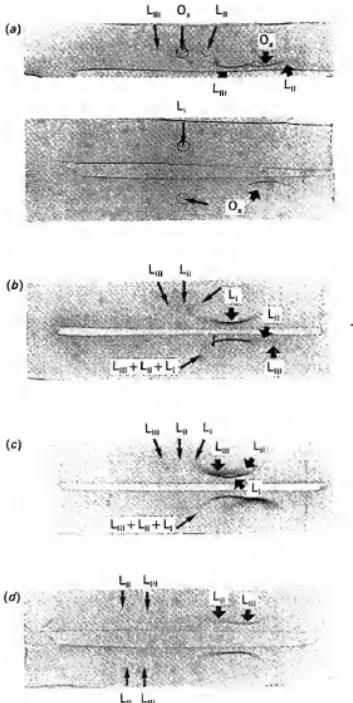


Fig. 2. Immunoelectrophoresis of *Phlebia* purified enzymes

Electrophoresis was in 50 mM-sodium barbital buffer, pH 8.2, on agarose gel. Protein staining was with Amido Black. Samples (1 µg) of purified oxidase, O_x, and lignin peroxidases L₁, L₂, L₃ were applied as shown by thin arrows. Enzymes after electrophoretic separation and immunoprecipitation are indicated by thick arrows. Antisera were diluted 1:2 and applied into the horizontal wells after electrophoresis. (a) Antiserum against O_x; (b) antiserum against L₁; (c) antiserum against L₂; (d) antiserum against L₃.

All the ligninolytic enzymes of *Phlebia radiata* were shown to be glycoproteins. Endoglycosidase H and F, by removing high-mannose heteroglycans, decreased the molecular sizes of *Phlebia* enzymes by 5–14%.

Lignin peroxidases of *Phanerochaete* are also glycoproteins, with neutral carbohydrate contents of 6% (w/w) (Renganathan *et al.*, 1985). *Polyporus* (Fähraeus & Reinhammar, 1967), and *Neuraspora* (Froehner & Eriksson, 1974) oxidases have been reported with 10–11% (w/w) carbohydrate.

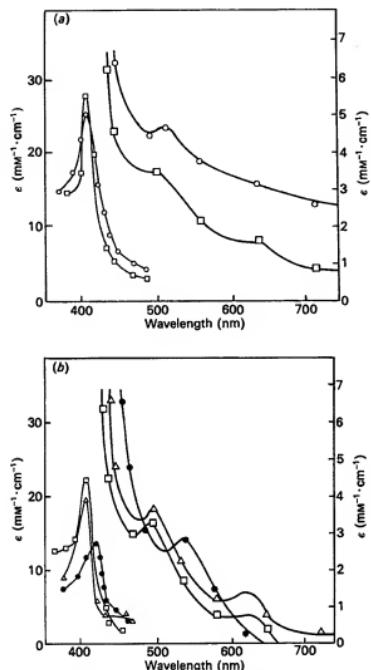


Fig. 3. Absorption spectra of L_{III} and its derivatives

(a) Spectra of 200 µg of L_{III} in 50 mM-sodium acetate buffer, pH 5.5, untreated (□) or modified by the sequential addition of 0.1 mM-dithiothreitol (○). (b) Spectra of 100 µg of L_{III} untreated (□) or modified by the addition of 0.1 mM- Na_2N_3 (△) or KCN (●). Spectra were measured over the range 300–700 nm immediately after addition of the supplement at 20 °C.

Lignin peroxidase I is serologically only partly identical with the other lignin peroxidases, L_{II} and L_{III} . The protein structure of L_I includes extra epitopes besides those in common with L_{II} and L_{III} . Serological similarities have recently also been found between the lignin peroxidases of *Phlebia* and those of *Phanerochaete* (Kanttilen et al., 1988).

The spectra of native lignin peroxidases of *Phlebia* and their derivatives strongly resemble those of animal catalase (Nicholls & Schonbaum, 1963), which contains protoporphyrin IX as a prosthetic group. Minor differences among L_I , L_{II} and L_{III} were revealed in the shifts of the absorption spectra obtained by reducing and oxidizing agents. This could indicate differences in the state of oxidation or in the ligands in the prosthetic

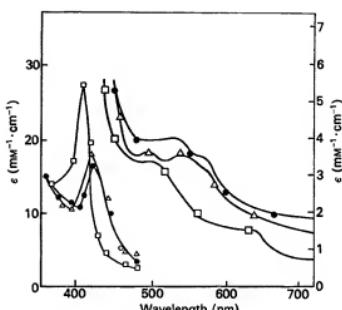


Fig. 4. Absorption spectra of L_{III} and its peroxide intermediates

Spectra of 200 µg of L_{III} in 50 mM-sodium acetate buffer, pH 5.5, untreated (□) or modified by the sequential addition of 0.15 mM- H_2O_2 (△) and 1.2 mM-veratryl alcohol (●). Spectra were measured immediately after addition of the supplement at 20 °C.

groups and hence explain the different ionic properties of *Phlebia* lignin peroxidases.

Spectral evidence shows that lignin peroxidases of *Phanerochaete* can achieve the five different redox states characteristic of conventional peroxidases (Tien & Kirk, 1984; Renganathan et al., 1985; Tien et al., 1986). With a molar equivalent of H_2O_2 , lignin peroxidase is converted into the one-electron-deficient Compound II, which shifts instantaneously back to the ferric ground state when veratryl alcohol is added as an electron donor. The spectrum of *Phlebia* lignin peroxidase shows a different regeneration cycle. After addition of veratryl alcohol *Phlebia* lignin peroxidase remains in the oxidized state for a longer period (Fig. 4). In catalase-mediated reactions Compound II is only slowly converted into the ground state. The formation of Compound III as a consequence of unbalanced stoichiometry between the reactants is also a possibility: the step from Compound III to the resting enzyme is slow for all peroxidase enzymes.

Resonance Raman spectral studies (Andersson et al., 1987) have shown that *Phanerochaete* lignin peroxidase more closely resembles horseradish peroxide (EC 1.11.1.7) than cytochrome c peroxidase (EC 1.11.1.5) or catalase (EC 1.11.1.6). The results obtained in the present work indicate that the lignin peroxidase of *Phlebia* is closer to catalase. However, definite conclusions concerning similarities between the *Phlebia* enzymes and other peroxidases require more data.

Phlebia oxidase is not a haemoprotein. It was shown to contain copper (atomic absorption analysis), although the spectral characteristics of known blue copper oxidases, including those of *Polyporus versicolor* laccase (Malkin & Malmström, 1970), were not exhibited. The featureless spectrum suggests the existence of copper as type 2 in *Phlebia* oxidase. Typical for type 2 copper in oxidase is inhibition by anions and tight binding with proteins. *Phlebia* oxidase was inhibited by anions and

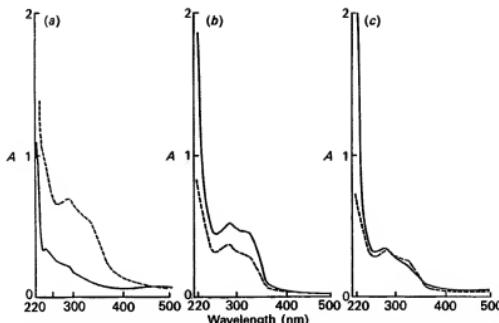


Fig. 5. Absorption spectra of Kraft lignin treated with ligninolytic enzymes

In each case 1 mg of Kraft lignin ($M_f < 1000$) was incubated with 10 μ g of enzyme at 20 °C for 10 min. (a) Incubation in 50 mM-dimethylsuccinate buffer, pH 4.5, with O_2 ; (b) and (c) incubation in 50 mM-glycine/HCl buffer, pH 3, containing 0.15 mM- H_2O_2 with L_n (b) and with L_m (c). Spectra of the buffer-soluble fraction from Kraft lignin before (----) and after (—) enzyme treatment are shown.

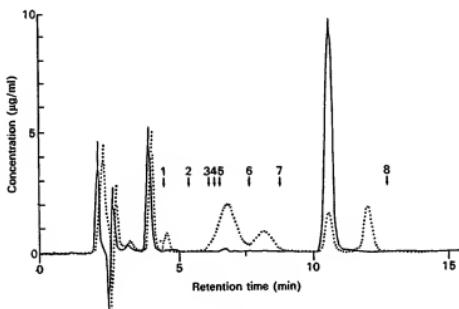


Fig. 6. H.p.l.c. analysis of guaiacol treated with *Phlebia* oxidase

Incubation conditions were as indicated for Fig. 5(a). Spectra of guaiacol before (—) and after (----) treatment with O_2 are shown. Retention of reference standards is indicated by arrows: 1, catechol; 2, veratryl alcohol; 3, coniferyl alcohol; 4, coumaric acid; 5, vanillin; 6, ferulic acid; 7, veratic acid; 8, veratraldehyde.

was not affected by EDTA. Further evidence for the Cu^{2+} form was insensitivity towards CO and inhibition by reducing agents. The absorption coefficients for type 2 copper compound are low, the limit of detection being 1 mM (Malkin & Malmström, 1970). Copper-containing extracellular laccases that lack the absorption spectra specific for *Polyporus laccase* have also been reported from *Agaricus bisporus* (Wood, 1980) and from *Schizophyllum commune* (de Vries *et al.*, 1986). *Phlebia* oxidase affected compounds containing a free phenolic hydroxy group. Such compounds included substituted *o*- and *p*-dihydroxybenzenes, trihydroxybenzenes and *p*-phenylenediamine, but not tyrosine or 3,4-dihydroxyphenylalanine

either separately or in combination. The reaction products obtained were generally coloured, suggesting the formation of quinones. The fate of O_2 was not analysed, but the reactions were strongly retarded when the incubation mixtures were purged with N_2 . On the basis of these results *Phlebia* oxidase is considered a benzenediol: O_2 oxidoreductase from the EC group 1.10.3.2. *Phlebia* oxidase did not cleave the aromatic ring of its substrate, nor did it affect two substrates simultaneously. The enzyme cannot therefore belong to the EC groups 1.13 and 1.14 (oxygenases, mono-oxygenases and mixed-function oxidases).

The pH optima for activity of *Phlebia* enzymes are

pH 3 and 4.5 for lignin peroxidases and for oxidase respectively, whereas the stability optima are at pH 5–6. The lignin peroxidases of *Phanerochaete chrysosporium* are also active at acid pH values, the optimum being near pH 2 and no activity being shown at pH values greater than 5.0 (Tien *et al.*, 1986). Haemoproteins are readily dissociated into haem and apoprotein at pH values below 4.0, and thus the pH optima for lignin peroxidases of *Phlebia* and *Phanerochaete* are rather surprising. On the other hand, the formation of cation radicals essential for lignin degradation is favoured at acid pH values.

Lignin peroxidases of *Phanerochaete* require slightly higher temperatures (approx. 45 °C) for optimal activity (Farrell, 1986) than do *Phlebia* enzymes (40 °C).

Several soil bacteria and fungi metabolize aromatic compounds by performing successive hydroxylation, decarboxylation, demethylation and oxidation reactions that together result in cleavage of the aromatic ring (Cain, 1980). However, a separate enzyme is involved for each step. Lignin peroxidases of the white-rot fungus *Phanerochaete* have been shown to be capable of all the reactions necessary for ring cleavage in lignin model compounds (Umezawa *et al.*, 1986). The biochemical function of ligninolytic enzymes from white-rot fungi is difficult to study because the amount of protein secreted is low, 0.01 g/l of culture liquid. For comparison, the amount of extracellular protein can be as high as 20 g/l with industrial enzyme producers such as *Trichoderma reesei* (Enari, 1983). To obtain purified ligninolytic components for industrial purposes, the enzyme production of white-rot fungi must be much improved.

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